Detection and characterization of *Clostridium perfringens* in the feces of healthy and diarrheic dogs

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Abstract

Clostridium perfringens has been implicated as a cause of diarrhea in dogs. The objectives of this study were to compare 2 culture methods and to evaluate a multiplex polymerase chain reaction (PCR) assay to detect *C. perfringens* toxin genes alpha (α), beta (β), beta 2 (β2), epsilon (ε), iota (ι), and *C. perfringens* enterotoxin (cpe) from canine isolates. Fecal samples were collected from clinically normal non-diarrheic (ND) dogs, (*n* = 105) and diarrheic dogs (DD, *n* = 54). *Clostridium perfringens* was isolated by directly inoculating stool onto 5% sheep blood agar (SBA) and enrichment in brain-heart infusion (BHI) broth, followed by inoculation onto SBA. Isolates were tested by multiplex PCR for the presence of α, β , β2, ε, ι, and cpe genes. *C. perfringens* was isolated from 84% of ND samples using direct culture and from 87.6% with enrichment (*P* = 0.79). In the DD group, corresponding isolation rates were 90.7% and 93.8% (*P* = 0.65). All isolates possessed the α toxin gene. Beta (β), β2, ε, ι, and cpe toxin genes were identified in 4.5%, 1.1%, 3.4%, 1.1%, and 14.8% of ND isolates, respectively. In the DD group, β and β2 were identified in 5%, ε and ι were not identified, and the cpe gene was identified in 16.9% of isolates. Enrichment with BHI broth did not significantly increase the yield of *C. perfringens*, but it did increase the time and cost of the procedure. *C. perfringens* toxin genes were present in equal proportions in both the ND and DD groups (*P* ≤ 0.15 to 0.6). Within the parameters of this study, culture of *C. perfringens* and PCR for toxin genes is of limited diagnostic usefulness due to its high prevalence in normal dogs and the lack of apparent difference in the distribution of toxin genes between normal and diarrheic dogs.

Résumé

Clostridium perfringens *a été impliqué comme agent de diarrhée chez les chiens. Les objectifs de la présente étude étaient de comparer deux méthodes de culture et d'évaluer une épreuve de réaction d'amplification en chaîne par la polymérase (PCR) multiplex permettant de détecter chez les isolats canins de* C. perfringens *les gènes des toxines alpha (α), beta (β), beta 2 (β2), epsilon (ε), iota (ι) et celui de l'entérotoxine (cpe). Des échantillons de fèces ont été obtenus de chiens cliniquement normaux et non-diarrhéiques (ND)* (n = *105) et de chiens diarrhéiques (DD,* n = *54).* Clostridium perfringens *était isolé en ensemençant les fèces directement sur des géloses supplémentées de 5 % de sang de mouton (SBA) et en procédant à un enrichissement dans un bouillon d'infusion de cœur-cervelle (BHI), suivi d'un ensemencement sur SBA. Les isolats ont été testés par PCR multiplex pour la présence des gènes* α, β, β2, ε, ι *et cpe.* Clostridium perfringens *a été isolé de 84 % des échantillons provenant des chiens ND par ensemencement direct et 87,6 % après enrichissement* (P = *0,79). Dans le groupe DD, les taux d'isolement correspondants étaient de 90,7 % et 93,8 % (*P = *0,65). Tous les isolats possédaient le gène de la toxine* α. *Les gènes des toxines* β, β2, ε, ι *et cpe ont été identifiés respectivement dans 4,5 %, 1,1 %, 3,4 %, 1,1 % et 14,8 % des isolats des chiens ND. Dans le groupe DD, les gènes des toxines* β *et* β2 *ont été identifiés à partir de 5 % des isolats, les gènes* ε *et* ι *n'ont pas été identifiés, et le gène cpe a été identifié chez 16,9 % des isolats. L'enrichissement avec le bouillon BHI n'augmentait pas de manière significative le taux d'isolement de* C. perfringens, *mais augmentait le délai et le coût de la procédure. Les gènes des toxines de* C. perfringens *étaient présents en proportions égales dans les groupes ND et DD (*P ≤ *0,15 à 0,6). En fonction des paramètres de la présente étude, la culture pour* C. perfringens *et l'analyse par PCR pour détecter les gènes des toxines sont d'une utilité diagnostique limitée étant donné la prévalence élevée chez les chiens normaux et le peu de différence apparente dans la distribution des gènes des toxines entre les chiens normaux et les chiens diarrhéiques.*

(Traduit par Docteur Serge Messier)

Introduction

Clostridium perfringens is an anaerobic, Gram-positive, spore-forming bacillus that has been implicated as a cause of diarrhea in both domestic animals and humans (1,2). *Clostridium perfringens* strains are classified into 5 toxigenic genotypes (A to E) on the basis of the production of 4 main toxins: alpha (α), beta (β), epsilon (ε), and iota (ι) (3,4). Additionally, individual *C. perfringens* strains can express up to 12 other toxin genes, including *C. perfringens* enterotoxin (cpe) and beta 2 toxin (β2) (5).

While *C. perfringens* is often considered an important canine enteropathogen, objective information about its role in disease is limited. No gold standard is yet available for diagnosing *C. perfringens*-associated diarrhea in dogs (5). Various methods are used, such as isolating *C. perfringens*, isolating *C. perfringens* with subsequent determination of type (toxigenic culture), detecting selected toxin

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genes in feces, and detecting toxin in feces, although the usefulness of these tests is not known. It is difficult to confirm the diagnosis of *C. perfringens*-associated diarrhea because the organism is ubiquitous in nature and is part of the endogenous flora of the canine gastrointestinal tract (3). Fecal cultures have played a significant role in diagnosing *C. perfringens*-associated diarrhea in dogs, particularly in dogs with hemorrhagic diarrhea accompanied by pyrexia and neutrophilia (6). The optimal culture methods for isolation of *C. perfringens* in dogs, however, have not been described.

The objectives of the present study were to compare 2 methods for culturing *C. perfringens:* Columbia sheep blood agar (SBA) culture and enrichment culture with brain-heart infusion (BHI) and to describe the prevalence and distribution of the genes encoding the *C. perfringens* toxins (α, β, β2, ε, ι, and cpe) using a multiplex polymerase chain reaction (PCR) on canine fecal isolates obtained from healthy and diarrheic dogs in a primary care setting.

Materials and methods

Before the study, fresh fecal samples were collected from dogs that presented to a primary care veterinary clinic in southern Ontario. Samples were collected serially, without selection, from 2 groups of dogs: clinically healthy, non-diarrheic (ND) dogs that were presented with stool samples collected for routine parasitological testing, and dogs presented with acute diarrhea for 5 to 7 d [diarrheic dogs (DD)]. All fecal samples were stored at −21°C. The ND group included all dogs with no history of diarrhea and with normal fecal consistency for the previous 30 d. In addition, the physical examination of these dogs was normal and they had not received antibiotic therapy or any other medical treatment in the previous 30 d. Dogs with a chronic history of gastrointestinal disease and those administered antimicrobial medications or probiotics in the previous 30 d were excluded.

Direct and enrichment broth cultures were both performed on the samples. For the direct method, the fecal sample was thawed to room temperature and a cotton-tip applicator was introduced into the fecal sample. A small amount of feces lightly coating the applicator tip was used to inoculate the feces directly onto 5% Columbia sheep blood agar (SBA) (BD, Franklin Lakes, New Jersey, USA) using a cotton-tip applicator and then streaked for isolation. The SBA was incubated in an anaerobic chamber at 37°C for 24 h. Presumptive identification of *C. perfringens* was based on the development of the characteristic double hemolytic zone on SBA. In suspect cases when a single hemolytic zone was observed, *C. perfringens* was identified by individually subculturing the colony in question on SBA and incubating the plates in an anaerobic chamber at 37°C for 24 h.

For the enrichment culture method, the fecal sample was thawed to room temperature and a cotton-tip applicator introduced into the fecal sample. A small amount of feces lightly coating the applicator tip was inoculated into 9 mL of BHI broth and incubated at 37°C for 24 h. Subsequently, 0.5 mL of the broth was inoculated onto SBA and incubated at 37°C in an anaerobic chamber for 24 h, with presumptive identification of *C. perfringens* as previously described.

For each sample, 1 or 2 isolates were tested for genes encoding the *C. perfringens* toxins (α, β, ε, ι, cpe, and β2) by multiplex PCR. Deoxyribonucleic acid (DNA) was extracted for *C. perfringens* isolates detected on SBA after incubation in the anaerobic chamber at 37°C for 24 h. Two to 3 *C. perfringens* colonies were suspended in 1 mL of sterile saline in a 1-mL Eppendorf tube. The solution was centrifuged at 12 000 × *g* for 60 s. The supernatant was removed and discarded. Next, 200 μL of Instagene Matrix (Bio-Rad, Hercules, California, USA) was added to the cellular pellet, which was then vortexed for 10 s. The solution was incubated at 57°C in a water bath for 1 h, vortexed for 10 s, and heated to 100°C in a heating block for 8 min. The solution was centrifuged at 12 000 × *g* for 3 min and the supernatant was removed and stored at −20°C until PCR was performed.

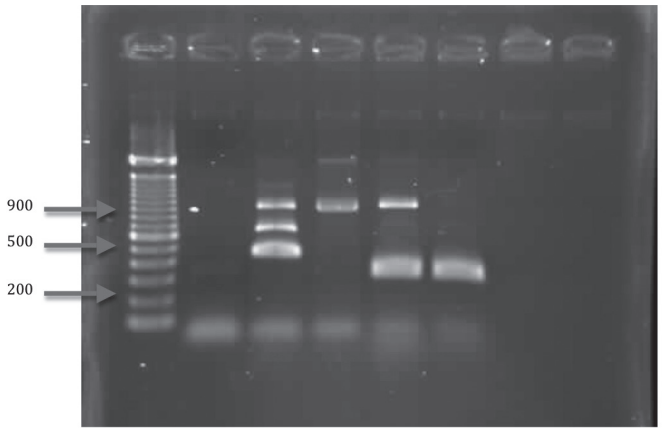

Figure 1. Multiplex PCR typing of *C. perfringens* toxin genes. Lane 1 1500 bp ladder, Lane 2 negative control, Lane 3 *C. perfringens* reference strain NCTC 3110, Lane 4 NCTC 8084, Lane 5 VN31-2 which has genes encoding for the alpa and β2 toxin gene.

Polymerase chain reaction (PCR) primers previously developed and validated by Baums et al (7) were used for the multiplex PCR reaction. The starting concentrations of *C. perfringens* primers CPA5L and CPA5R were 0.4 pM/μL. The amplification product for the various genes was: cpa gene (α toxin) — 324 bp in length; cpb gene (β toxin) — 195 bp; cpb2 gene (β2 toxin) — 548 bp; etx gene (ε toxin) — 376 bp; iap gene (ι toxin) — 272 bp; and cpe gene (enterotoxin) — 485 bp.

The 40-μL PCR reaction mixture included the following components: a total of 4 μL of primers for the 6 toxin genes; 5 μL of dNTP (Invitrogen, Carlsbad, California, USA); 1.5 μL of 50 mmol/mL of magnesium chloride (Invitrogen); 5 μL of buffer solution (Invitrogen); 0.5 μL of *Taq* DNA polymerase (Platinum *Taq* Polymerase; Invitrogen); and 24 μL of sterile water. The reaction mixture was combined with 10 μL of extracted DNA. Negative and positive controls were used for each PCR run. The negative control included 10 μL of PCR-grade water instead of the DNA from the clinical samples, whereas the positive control included 10 μL of NCTC 3110 reference strain (National Culture Type Collection, Health Protection Agency, London, UK). The PCR assays were performed using the MyCycler Thermal Cycler system (Bio-Rad).

The PCR program was as follows: initial denaturation for 15 min at 95°C, followed by 40 cycles each of denaturation for 30 s at 94°C, annealing for 90 s at 53°C, and extension for 90 s at 72°C, and final extension for 10 min at 72°C. This was followed by electrophoresis of 10 μL of the amplified products on a 2.0% agarose gel, staining with ethidium bromide, and visualization under ultraviolet (UV) light (Figure 1) (*C. perfringens* toxin genes on 1% agarose gel.) The following 3 *C. perfringens* reference strains were tested: NCTC 3110 9

Table I. Comparison of culture methods for *C. perfringens* in dogs

Group	Direct culture	Enrichment culture	*P*-value
Non-diarrheic (ND)	88/105 (84%)	92/105 (88%)	0.74
Diarrheic (DD)	58/65 (89%)	61/65 (94%)	0.67
P-value	0.64	0.13	

(National Culture Type Collection), which has genes encoding the α, β, and ε toxins; NCTC 8084 (National Culture Type Collection), which has genes encoding the alpha and ι toxins; and VN31-2 (obtained from retail chickens isolated by Vicki Nowell in Dr. John Prescott's laboratory, University of Guelph, Ontario), which has genes encoding alpha and β2 toxins. All diarrheic samples from which an adequate volume of stool remained were tested for cpe by performing a *C. perfringens* enterotoxin enzyme-linked immunosorbent assay (ELISA) (Tech Lab, Blacksburg, Virginia, USA) according to the manufacturer's instructions. This research was approved by the Animal Care Committee at the University of Guelph.

Statistical methods

McNemar's test for correlated percentages was used to compare the agreement between the 2 culture methods. Fisher's exact test was used to determine the difference between healthy and diarrheic dogs.

Results

From September 2009 to April 2010, 170 fecal samples were collected, 105 from healthy non-diarrheic dogs and 65 from diarrheic dogs. The median age of dogs in the ND and DD groups was not statistically different and was 4.2 y (from 0.6 to 12 y) and 3.9 y (0.8 to 13 y), respectively ($P = 0.91$). The HD group included 5 intact male dogs, 49 neutered male dogs, 4 intact female dogs, and 47 spayed female dogs. The DD group included 4 intact male dogs, 44 neutered male dogs, 2 intact female dogs, and 15 spayed female dogs. No significant differences were observed between the HD and DD groups with respect to gender or breed.

Culture results and associated *P*-values are presented in Table I. There was no difference in isolation rates between either the 2 culture methods or groups. All isolates possessed α toxin gene (Table II). Other toxin genes were uncommonly identified and there was no statistically significant difference between the 2 groups with respect to toxin genes identified (Table II).

There were 45 diarrheic fecal samples available for detection of cpe by ELISA. *Clostridium perfringens* enterotoxin (cpe) was detected in 5 of 45 samples (11%). All of the 5 samples that were cpe positive (100%) harbored the cpe gene detected by multiplex PCR; 39 of the 49 samples (79%) were negative for both cpe by ELISA and the cpe gene [sensitivity 83%, confidence interval (CI), 0.44 to 0.97, specificity 100%; CI, 0.91 to 1].

Table II. *Clostridium perfringens* toxin genes detected in SBA isolates from ND and DD groups

C. perfringens toxin gene	Non-diarrheic dogs (ND)	Diarrheic dogs (DD)	*P*-value
α	88/88 (100%)	58/58 (100%)	0.64
β	4/88 (4.5%)	3/58 (5%)	0.74
ε	3/88 (3.4%)	0/58 (0%)	1.00
ι	1/88 (1.1%)	0/58 (0%)	1.00
cpe	13/88 (14.8%)	10/58 (17.2%)	0.78
β2	1/88 (1.1%)	3/58 (5%)	0.53

Discussion

In this study, *C. perfringens* was readily cultured directly onto SBA and the use of enrichment culture techniques did not significantly increase the yield of *C. perfringens*. The high prevalence of *C. perfringens* in both healthy and diarrheic dogs is consistent with previous studies and further supports the finding that this organism is a common commensal in dogs.

Given the high prevalence and lack of a significant increase with enrichment, direct culture should be adequate for most studies investigating the role of *C. perfringens* in canine diarrhea. Enrichment culture techniques have been successfully used for obtaining *C. perfringens* isolates (8–10). It is difficult to justify the use of enrichment for routine study, however, as it takes longer, (11), costs more, and creates a greater potential for contamination.

The identification of *C. perfringens* was based solely on the presence of the characteristic double-hemolytic zone. Although the phenotypic identification was in 100% agreement with subsequent PCR, the possibility of false negative or positive results cannot be ruled out. Subsequent testing of each isolate for further identification would decrease the potential for such results.

This study did not compare different direct culture methods. A variety of selective and non-selective agar is available as well as other approaches such as the use of alcohol or heat shock methods to select for spores (12). The high yield in this study with non-selective SBA suggests that this relatively inexpensive, easily accessible culture medium is adequate. Despite a large number of SBA plates having considerable competing growth, *C. perfringens* isolates were identified readily on visual inspection and identification was confirmed by the presence of the alpha toxin gene (cpa).

As this study investigated fecal samples collected at a single urban veterinary clinic, care should be taken in extrapolating prevalence data to a broader population. The study does, however, provide some insight into the prevalence of *C. perfringens* and its toxin genes in a population of dogs at a primary care veterinary facility in southern Ontario.

With the high prevalence of *C. perfringens* in healthy dogs and no difference between healthy and diarrheic dogs, this study provides further support for the lack of clinical usefulness of culture for diagnosing *C. perfringens*-associated diarrhea in dogs. Molecular

diagnostic testing is increasingly available and numerous commercial assays are marketed for diagnosing diarrhea in dogs. The results of this study, however, do not support the usefulness of PCR assays, at least as the sole method, for diagnosing *C. perfringens*-associated diarrhea in dogs since no differences were identified between diarrheic and non-diarrheic dogs.

As expected, all fecal isolates possessed the alpha toxin gene (cpa) that is present in all types of *C. perfringens.* Data from this and other studies indicate that PCR assays targeting cpa should almost always be positive and of virtually no clinical use (8). The role of other toxins in canine diarrhea remains unclear. Previous studies have focused mainly on detecting the cpe gene and cpe ELISA as cpe has been implicated as a cause of canine diarrhea (8,13–15). There is a small amount of published data determining the distribution of the genes encoding the toxins α, β, ε, ι, and β2 in a population of normal and diarrheic dogs (8). In the current study, the cpe gene was detected in 14.4% of healthy dogs, which is consistent with the results of previous studies (3,8,10). As the presence of the cpe gene does not imply its expression, the prevalence may represent the normal carriage of the cpe gene in this population of dogs, but may not necessarily provide evidence of its role in the development of diarrhea. Although the number of cpe toxin gene-positive samples tested using the enterotoxin ELISA was small, this test appears to be specific (100%) as none of the samples was negative for the cpe toxin gene and ELISA positive.

Although compared to PCR detection of cpe, the sensitivity of the ELISA assay used in this study was moderate (83%), care must be taken when interpreting sensitivity because of the small number of positive samples and the wide confidence intervals. Further assessment of the cpe ELISA assay is required based on these encouraging preliminary results. Despite the strong association between the cpe-positive samples and cpe ELISA-positive samples, the role of enterotoxin in the development of canine diarrhea remains unclear because 14% of the HD fecal samples were also positive for the cpe toxin gene. It has been suggested that the optimum diagnostic approach would include the use of an ELISA for detecting *C. perfringens* enterotoxin in conjunction with the use of PCR for detecting enterotoxigenic strains (16). This recommendation has been based on concern about the sensitivity and specificity of the cpe ELISA, although the preliminary results of this study may suggest that this may not be needed. A larger prospective study comparing the results of cpe PCR and cpe ELISA is required to further investigate the role of enterotoxin in the development of canine diarrhea.

Recently, the novel β2 toxin has been implicated as a cause of necrotizing enterocolitis in many species, including dogs (2,17,18). The prevalence of the β2 toxin in healthy dogs has previously been reported in up to 15% of healthy dogs and in 22% to 33% of diarrheic dogs (3,15). In the current study, the β2 toxin gene was detected in 1.2% of healthy dogs and in 5% of diarrheic dogs. The presence of the β2 toxin gene in diarrheic fecal samples does not implicate it as a cause of diarrhea because it is not known whether the gene was expressed in these samples or whether toxin production is associated with development of clinical disease. In a study by Thiede et al (15), the cpb2 was detected in conjunction with the cpe in diarrheic samples. Despite the small number of dogs included in this study, the authors hypothesized that cpb2 either had a direct pathogenic role in the development of canine diarrhea or it worked synergistically with cpe, as both cpb2 and cpe were detected in 16% of the isolates used in the study (15). None of the samples in the current study was positive for both cpe and cpb2.

Clostridium perfringens β, ε, and ι toxins can cause disease in many domestic animals. *Clostridium perfringens* has been identified as the causative agent in dysentery and enterotoxemia (pulpy kidney disease) in lambs and in enteritis necroticans (pigbel) (1). There is little information available about the prevalence of these toxin genes in healthy and diarrheic dogs and no difference was noted between the presence of these toxin genes in healthy and diarrheic dogs in the present study. Interestingly, the epsilon toxin gene (etx) and the iota toxin gene (iap) were detected in 3.3% and 1.1%, respectively, of healthy dogs, but were not detected in any of the diarrheic dogs. Due to the small sample size, the prevalence of these genes may have been underestimated, but their absence brings into question their overall role in the development of *C. perfringens*-associated diarrhea in dogs. In addition, only 1 to 2 isolates were tested per sample, which could alter the true prevalence of the toxinotypes in the population tested. It is possible that not all isolates from a single dog have the same toxinotype, which may have decreased the presence of specific toxin genes. Clearly, the presence of *C. perfringens* carrying these genes is not strictly associated with the presence of diarrhea, which suggests that toxigenic culture or PCR detection of these genes are of limited diagnostic utility.

In conclusion, both cultured *C. perfringens* and *C. perfringens* toxin genes were present in equal proportions in healthy and diarrheic dogs. Direct culture methods were just as good as enrichment techniques for isolating *C. perfringens,* but decreased the time and expense when isolating *C. perfringens.* The high prevalence of *C. perfringens* cultured from healthy dogs further obscures the role of *C. perfringens* in canine diarrhea. In addition, *C. perfringens* culture and PCR for detecting toxin genes have limited usefulness when used individually as diagnostic tests.

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